Antimicrobial resistance and class 1 integrons in *Salmonella enterica* subsp. *enterica* serovar Derby isolates from pig abattoirs

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Abstract
Salmonella enterica subsp. *enterica* (*S.*) serovar Derby is one of the most prevalent serovars in pigs. The aim of this study was the investigation of *S.* Derby isolates for the presence of antimicrobial resistance and class 1 integrons and their gene cassettes. Forty-nine *S.* Derby isolates, obtained from different sources at four pig abattoirs (A-D) in Southern Brazil were analysed. Five isolates were susceptible to all antimicrobial agents tested. Twenty-seven isolates were multi-resistant, showed a common resistance pattern to streptomycin/spectinomycin-sulphonamides-tetracycline and shared the same XbaI-pattern. Except for one isolate, all these multi-resistant isolates carried class 1 integrons. The integrons are most likely located in the chromosomal DNA of 16 and ten *S.* Derby isolates from abattoirs A and B, respectively. All amplicons for the variable part of class 1 integron showed the same EcoRI RFLP pattern. The integrons harboured a new *aadA* variant designated *aadA26*, which encodes combined resistance to streptomycin and spectinomycin. The presence of the same class 1 integron among related isolates, from the same or different abattoirs, points towards a dissemination of the integron by a clonal expansion of the isolates.

Introduction
Salmonella enterica subsp. *enterica* serovar (*S.*) Derby has been commonly isolated from slaughter pigs and pork products (Hauser et al., 2011). In 2011, *S.* Derby was among the top five serovars most frequently isolated from clinical and non-clinical isolates from non-human sources, which were submitted to the National Veterinary Services Laboratories (NVSL) in the U.S.A. Moreover, it was the second most isolated serovar from porcine sources (CDC, 2013). In Southern Brazil, this serovar was also among the most common serovars isolated from pigs and pork products (Bessa et al., 2007; Mürmann et al., 2009).

Multi-resistant (resistance to three or more classes of antimicrobial agents) *S.* Derby isolates have been obtained from different sources, and integrons with different gene cassette arrays have been identified in this serovar (Akiba et al., 2006; Beutlich et al., 2011). Integrons are genetic elements able to integrate and excise gene cassettes by site-specific recombination: They usually carry antimicrobial resistance gene cassettes and therefore contribute to maintenance and dissemination of antimicrobial resistance. The aim of this study was the investigation of *S.* Derby isolates from pigs for (a) the presence of antimicrobial resistance, (b) the detection of class 1 integrons and their gene cassettes and (c) the location of the class 1 integrons.

Material and Methods
A total of 49 *S.* Derby isolates obtained from lairage, pig carcasses and intestinal contents at four abattoirs (A-D) in 2008 in Southern Brazil were analyzed. They were tested for susceptibility to 12 antimicrobial agents by agar disk diffusion (CLSI, 2008). Multi-resistant isolates (n=27) were further investigated by XbaI-macrorestriction analysis (Ribot et al., 2006), plasmid profiling (Schwarz and Liebisch, 1994), and PCR assays for the detection of the resistance genes: *sul1*, *sul2* and *sul3* (sulphonamide resistance), *tet*(A) and *tet*(B) (tetracycline resistance) and *strA* (streptomycin resistance) and *aadA* variants (streptomycin/spectinomycin resistance) (Frech et al., 2003; Kadlec et al., 2005).

Integrons were screened by PCR assays for the presence of the *intI1* integrase gene and the variable part of class 1 integrons (Sandvang et al., 2007). The amplicons specific for the variable part of class 1 integrons were analysed by restriction fragment length polymorphism (RFLP) using the EcoRI restriction enzyme. A representative amplicon was chosen for cloning into pCR®2.1-TOPO Vector (Invitrogen, Groningen, The Netherlands) and the recombinant vector was transformed into *Escherichia coli* recipient strain TOP10. Sequence analyses were conducted with the M13 forward and reverse primers (MWG, Ebersberg, Germany). Sequence comparisons were carried out using the BLAST programs blastn and blastp (http://www.ncbi.nlm.nih.gov/BLAST/) and with the ORF Finder program (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The nucleotide sequence of the amplicon for the variable part of class 1 integron has been deposited in the European Molecular Biology Laboratory (EMBL) database under the accession number HG314953.1. To confirm the linkage between *aadA26* and *sul1*, as well as, *aadA26* and *qacE*Δ1, specific PCR assays were used (Michael et al., 2005).

The location of the class 1 integrons was determined by Southern blot hybridization. For this, plasmid DNA was prepared by alkaline lysis and whole-cell DNA was digested using EcoRI restriction enzyme. The plasmid and whole-cell DNA were transferred from agarose gels to a nylon membrane by the capillary blot procedure. A sequenced amplicon for the variable part of the class 1 integron was enzymatically labelled by the Dig-Hib prime DNA labelling and detection system (Roche, Mannheim, Germany) and used as probe. Hybridization and signal detection were carried out according to the manufacturer's recommendations.

Results

Of the 49 *S*. Derby isolates originally tested, five isolates were susceptible to all antimicrobial agents tested and 27 isolates showed multi-resistance to streptomycin/spectinomycin, sulphonamides and tetracycline. The multi-resistant isolates were found in samples from lairage, pig carcasses and intestinal contents or from pig carcasses and intestinal contents at abattoir A (n=16) or B (n=11), respectively. Among the multi-resistant isolates five plasmid profiles and an indistinguishable XbaI-macrorestriction pattern were seen. Moreover, PCR analysis revealed that all multi-resistant isolates carried an *aadA* variant gene coding an aminoglycoside adenyltransferase which confers combined resistance to streptomycin/spectinomycin, the gene *sul1* coding for a sulphonamide-resistant dihydropteroate synthase and the tetracycline exporter gene *tet*(A). The PCR screening for class 1 integrons identified the class 1 integrase gene *intI1* in 26 isolates (abattoir A, n=16 and abattoir B, n=10). In addition, the PCR for the variable part of the integron showed an amplicon of approximately 1 kb. Restriction analysis of amplicons revealed the same fragment patterns in all isolates consisting of two EcoRI fragments of 560 and 449 bp. A representative amplicon was cloned and sequenced.

Sequence analysis of the class 1 integron confirmed the presence of a 1009 bp amplicon of which the first 118 bp were part of the 5' conserved segment (CS) and the final 111 bp were part of the 3' CS of the class 1 integron. The variable part (780 bp) showed a single gene cassette with a reading frame coding for a new variant of the AadA aminoglycoside adenyltransferase, designated AadA26 (Fig 1). The *aadA26* gene cassette showed an amino acid substitution in the coding region, IIe® Val at the position 209, which is unique in the databases (NCBI database last accessed date: 11.07.2013). The *aadA26* gene has a putative GTG translational start codon (positions 119-121) and codes for an aminoglycoside 3'-(9)-O-adenyltransferase of 259 amino acids. This gene cassette proved to be functionally active for combined resistance to streptomycin and spectinomycin. The 59-base element of the gene cassette was identified containing the binding sites 1L, 2L, 1R and 2R for the *intI1*-encoded integrase. The linkage of *aadA26* to *sul1* or *aadA26* to *qacE*Δ1 was confirmed by the specific PCR assays for all isolates carrying class 1 integron. Since the hybridization experiments did not yield signals when using plasmid DNA as targets for the cassette-specific probe, the class 1 integrons are probably located in the chromosomal DNA of the *S*. Derby isolates. Additional studies are on-going to determine the location of the class 1 integrons in the chromosomal DNA and to investigate if the 3'-CS or 5'CS regions are absent in the single multi-resistant isolate, which was not positive for the detection of *intI1* gene and the variable part of class 1 integrons.

Discussion

In the present study, the same class 1 integron was detected in multi-resistant *S*. Derby isolates obtained during a survey study at two pig abattoirs in Southern Brazil. The same class 1 integron was detected in related isolates from different sources and different abattoirs, which may suggest the spread of a resistant clone of *S*. Derby in the pig production chain in the Southern of Brazil. An explanation for this clonal dissemination, within this particular geographic area in Brazil, could be the pig production system. It is a vertically integrated system, in which the abattoirs are supplied by specific finishing farms that purchase the piglets from common pig farms. Moreover, the feed is also supplied by a common feed industry. Interestingly, the multi-resistance pattern (streptomycin /spectinomycin-sulphonamides-tetracycline) found with these isolates was also the most common pattern found in a previous study (sampling period 1999-2000), in which 24 porcine *S*. Derby isolates from Southern Brazil were characterized (Michael et al, 2006). Comparing these two studies, almost all isolates that shared the same resistance phenotype showed also the same resistance genotype, except a single *sul2* resistance gene and the *aadA* variant of the gene cassette of the class 1 integrons. In the previous study, an *aadA2* variant was found (Michael et al., 2005) and in the present study a new variant, *aadA26*, was identified. The *aadA26* can be distinguished from *aadA2* variant by a single amino acid substitution. Moreover, the isolates from the present study shared the same XbaI macrorestriction pattern with one isolate or differed by only one fragment from another 23 isolates of the previous study (Michael et al, 2006). Although there is a 8-9 years interval between the two survey studies, the class 1 integrons and their gene cassettes proved to be highly conserved and stable. The association between the presence of class 1 integron and tetracycline resistance may be due to genetic linkage of integrons and transposons carrying the gene *tet*(A). Although streptomycin and sulphonamide have not been administered to pigs in Southern Brazil anymore, and the use of tetracycline has been steadily declining in the last years, such resistance determinants are likely to persist in the *Salmonella* population.

Conclusions

Class 1 integrons are present in *S*. Derby isolates from apparently healthy pigs at slaughter and seem to represent genetic elements that are highly conserved and stable in this population in Southern Brazil. Moreover, the presence of the same class 1 integron among related isolates, from the same or different abattoirs, points towards a dissemination of the integron by a clonal expansion of the isolates in apparently healthy pigs. Noteworthy, such asymptomatic carriers may promote the dissemination of *S*. Derby not only to other animals, but also to humans when the isolates enter the food chain.

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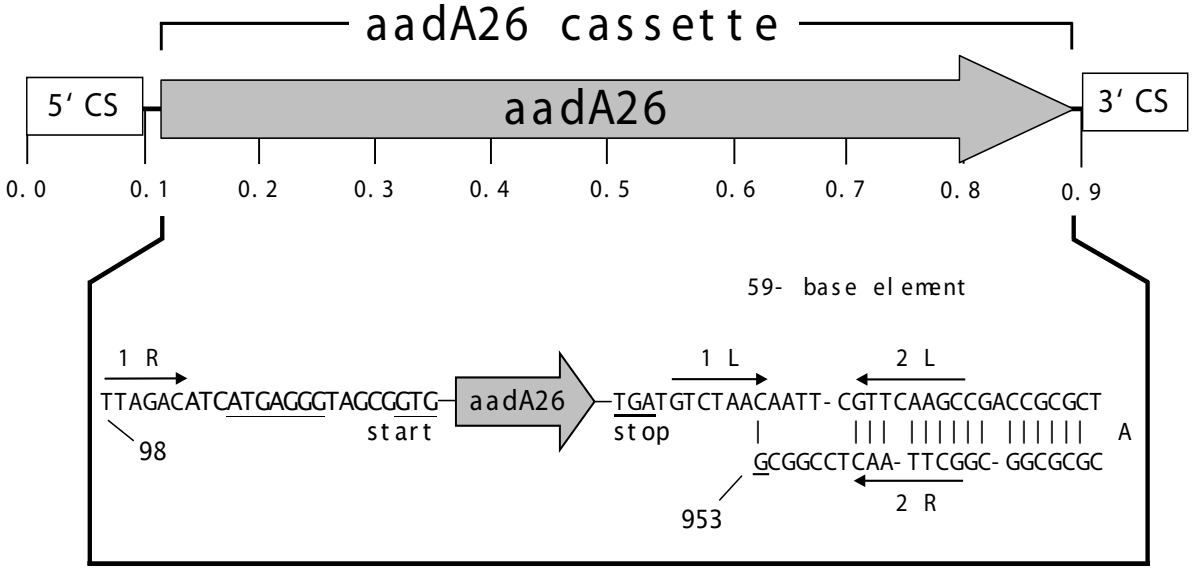

Fig. 1 Schematic presentation of the *aadA26* gene cassette from *S*. Derby. The *aadA26* reading frame is shown as an arrow whereas the 5' and 3' conserved segments (5' CS, 3' CS) of the class 1 integron are displayed as boxes. The beginning and the end of the gene cassette are shown in detail below. The translational start (GTG) and stop (TAA) codons are underlined. The 59-base element is presented as a stem-loop structure and the integrase 1 binding domains 1L, 2L, 2R, and 1R are indicated by arrows. The 59-base element of the gene cassette is shown in bold type. Numbers indicating important positions of bases in the 59-base element refer to the corresponding database entry (accession no. HG314953.1).